Critical self-association of bile lipids studied by infrared spectroscopy and viscometry

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Abstract Fourier transform infrared (FTIR)-attenuated total reflection (ATR) spectroscopy and viscometry were applied to study the micellization of two bile lipids, sodium taurochenodeoxycholate (NaTCDC) and sodium glycocholate (NaGC), in aqueous solutions. The CH_2 stretching bands of the bile lipid hydrocarbon region were shifted to higher frequencies suggesting initial critical micellization at 2.5 mM for NaTCDC and 9 mM for NaGC. An abrupt enhancement of the absorption intensity of the CH_3 groups of the sterol rings in bile lipids were under conformational strain at 3.5 mM NaTCDC and 9 mM NaGC. Viscometry measurements showed abrupt changes in viscosities in the region of critical micellar concentration (CMC) of both bile lipids. Both infrared and viscometry studies confirmed the onset of conformational strains in tightly packed lipid micelles at their CMC. In addition, FTIR/ATR spectroscopy has defined the specific hydrophobic interactions which bring about critical micellization of bile lipids.—**Antonian, L., S. Deb, and W. Spivak.** Critical self-association of bile lipids studied by infrared spectroscopy and viscometry. *J. Lipid Res.* 1990. **31:** 947–951.

Supplementary key words critical micellization concentration • Fourier transform infrared spectroscopy • sodium taurochenodeoxycholate • sodium glycocholate

In aqueous solutions bile salt monomers form globular aggregates, namely micelles, with nonpolar hydrophobic interiors and polar hydrophilic surfaces facing the aqueous environment. Bile salts have large hydrophobic regions and with increasing monomer concentration, self aggregate in order to avoid contact with water. Over a narrow concentration range, referred to as the critical micellar concentration (CMC), some bile salts exhibit critical self-association with a strong cooperative interaction of a number of bile salt monomers to form a micelle (1).

We have studied those physical chemical features that affected the CMC formation of NaTCDC and NaGC, by using infrared (IR) spectroscopic and viscometric methods. Since IR spectroscopy measures vibrational properties of molecular bonds, it is well suited for the study of the interaction of lipids. However, IR spectroscopy of biliary lipids in aqueous solutions has been hampered because of the intense absorption of water. We have overcome this problem and have measured the IR spectra of bile salts in water by using FTIR in conjunction with attenuated total reflectance (ATR) cylindrical cell. Infrared absorbance spectra of bile lipids were dominated by characteristic absorbance pattern of the aliphatic stretch region, especially during critical micellization process. The spectroscopic properties of CMC formation were also corroborated by highly sensitive viscometric measurements. Since the formation of micelles is associated with an increase in the hydrodynamic radius (Rh) of aggregates in solution, and since viscometric methods are sensitive to changes in Rh, we were able to measure small but significant changes in viscosity during micellar formation.

MATERIALS AND METHODS

Chemicals

NaTCDC (98% pure by TLC and elemental analysis) and NaGC (98% pure) were purchased from Sigma Chemical Co. All glassware was acid-washed and rinsed thoroughly with distilled water before use. NaTCDC and NaGC solutions were prepared in 200 mM NaCl. The pH of the NaTCDC solution ranged from 6.04 to 6.23 as its concentration was increased from 1 mM to 5 mM. The pH of the NaGC solution in sodium chloride solution ranged from 6.14 to 6.52 as its concentration was increased from 4 mM to 14 mM. NaGC was also prepared in 100 mM Na phosphate buffer at 8.0.

Abbreviations: CMCC, critical micellar concentration; FTIR, Fourier transform infrared spectroscopy; ATR attenuated total reflection; NaTCDC, sodium taurochenodeoxycholate; NaGC, sodium glycocholate; Rh, hydrodynamic radius; BMG, bilirubin IXα monoglucuronide; SCMC, super critical micellar concentration; TLC, thin-layer chromatography.

FTIR methods

IR spectra of biliary bile salt solutions were obtained by using a cylindrical internal reflectance cell (CIRCLE cell, SpectaTech, Stamford, CT) with a ZnSe crystal and a 1 ml internal volume. Spectra were acquired on an Analect RFX-30A FTIR spectrometer (Laser Precision Analytical, Irvine, CA) equipped with a TGS detector and interfaced with a Compaq 386 computer. Both the IR source and sample compartments were continuously flushed with argon in order to minimize background absorptions of CO_2 and H_2O. In order to achieve a satisfactory signal-to-noise ratio, 512 spectra were acquired at a resolution of 4 cm^{-1}. Apodization was performed with a medium Norton-Beer function; the spectral range 4400 to 750 cm^{-1} was stored for further processing. The spectra of each aqueous buffer was subtracted from the spectra of the corresponding bile salt solution, thereby correcting for the strong absorption of water. The aliphatic stretch region (2700–3100 cm^{-1}) of biles and the sulfate stretch region (1170–1210 and 1080 cm^{-1}) in TCDC were easily detected in the corrected spectra.

All corrected spectra were analyzed using SpectraCalc (Galactic Industries Corp., Salem, NH) a computer program designed for mathematical analysis of spectra. Peak positions and peak widths were assigned by a curve-fitting program within SpectraCalc. SpectraCalc was primarily used for determination of peak wave numbers. Deconvolution and smoothing of complex mixtures of peaks were subsequently performed merely for simplifying graphical representations (e.g., noise reduction, as shown in Figs. 1 and 5).

Viscosity methods

The viscosities were determined using an Irvine-Park Falling Needle Viscometer Model FNV100 (J&L Instruments Corp., Instruments Corp., King of Prussia, PA). The bile salt solution was placed in a precision inner quartz tube, surrounded by circulating water at a constant temperature of 26°C. A cylindrical glass needle of known density was dropped 4 cm through the test solution, and the drop time between two marked points was noted (precision for drop time was >95%). All measurments were preformed in triplicate and the mean was determined. The drop time was measured for various concentrations of bile salts. The densities of the bile salt solution were measured by weighing a known volume of the solution at 26°C. Viscosity was derived from the following equation relating viscosity to drop time and density of the test solution:

$$\mu = \frac{(\delta\sigma - \delta n)g}{U_t G}$$

where μ = viscosity; δ_n = density of needle; $\delta\sigma$ = density of solution; g = gravitational constant; U_t = terminal velocity (cm/sec); and G = geometric constant of the needle (specified by J&L Insturments Co.). The viscosity of the solution was then plotted against the concentrations of bile salt.

RESULTS

Micellar-induced structural changes in bile lipids were studied by monitoring the frequency and intenstiy of various infrared absorption bands at increasing bile salt concentrations. The transition from the monomer to micellar phase produced considerable changes in the aliphatic stretch region (2700–3100 cm^{-1}) of the IR spectrum.

Three distinct spectral changes were seen in the region of the CMC of NaTCDC. First, frequency and intensity increases were observed in the CH_2 symmetric (2865 cm^{-1}) and asymmetric (2939 cm^{-1}) stretching regions (**Fig. 1**). Frequency changes of the cymmetric CH_2 stretch were small for 1–5 mM NaTCDC and therefore were not indicative of CMC formation (figure not shown). In contrast, the CMC of NaTCDC was characterized by significant frequency changes of the asymmetric CH_2 stretching bands between 1 and 3 mM NaTCDC (**Fig. 2**). Secondly, the CH_3 asymmetric (2970 cm^{-1}) band emerged at 3 mM and became most prominent at 3.5 mM NaTCDC (Fig. 1), suggesting tightly packed bile salt molecules where the axial methyl groups of the sterol ring are sterically hindered. There also was a sharp increase in the absorption intensity at 3.5 mM. The continuous intensity increases

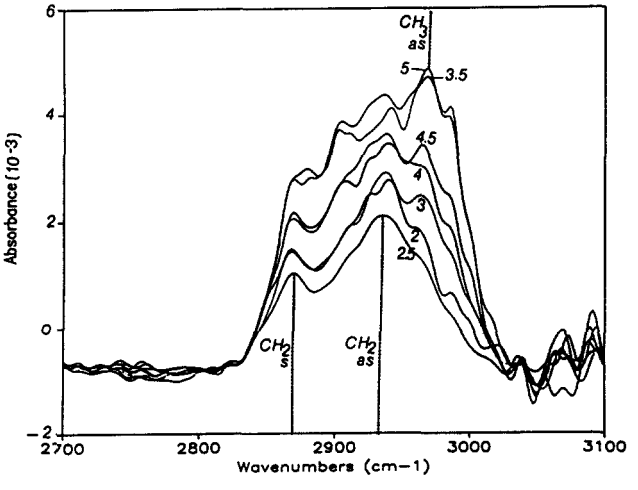

Fig. 1. IR spectra of 2–5 mM NaTCDC in 200 mM NaCl shows that spectral changes occur in the region of the CMC. 1) Frequency and intensity increases occur in CH_2 symmetric (CH_{2s}, 2865 cm^{-1}) and asymmetric (CH_{2as}, 2939 cm^{-1}) stretching regions. 2) The CH_3 asymmetric stretch (2970 cm^{-1}) emerges at 3 mM and is most prominent at 3.5 mM NaTCDC, suggesting a tightly packed micellar structure.

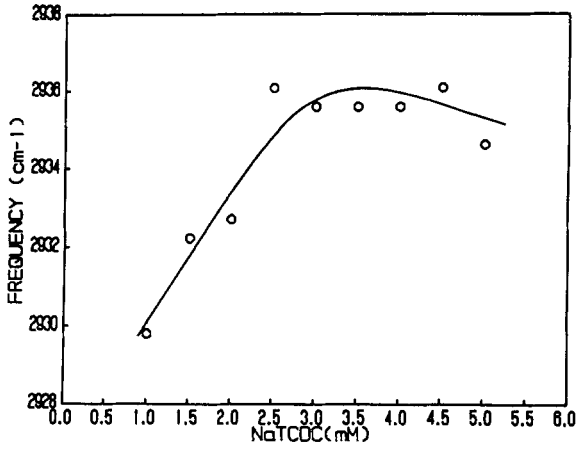

Fig. 2. Infrared absorbance maxima of the asymmetric CH_2 stretching bands 1-5 mM NaTCDC in 200 mM NaCl, at pH 6.1. The CMC is characterized by significant frequency changes between and 1 and 3 mM. The graph shows the average from three experiments and the standard error was <4 cm^{-1}.

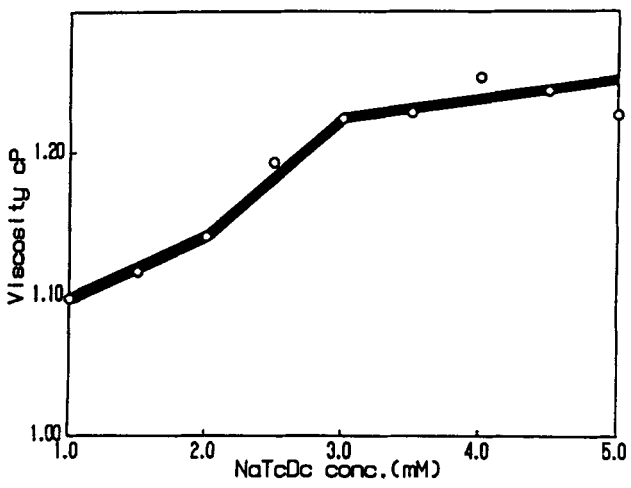

Fig. 3. Viscosity measurements of 1-5 mM NaTCDC in 200 M NaCl. CMC formation in this representative graph is noted by an abrupt increase in viscosity between concentrations of 2.5-3.5 M.

observed for all other concentrations, however, were approximately proportional to the increasing bile salt concentrations. Since the sulfonate head groups are not primarily involved in micellization (2), concentration-dependent changes in the absorbance frequencies of the $SO3^-$ group of NaTCDC were not present. However, at 3.5 mM NaTCDC an increase in absorbance intensity of the sulfonate symmetric stretch at 1080 cm^{-1} was present, similar to the sudden absorption increase in aliphatic region at the same concentration. This jump in the intensity of the sulfonate stretch region could be the result of a tightly packed aliphatic region which could bring the sulfonate head groups closer together. These IR absorption data suggest that maximal hydrophobic and head group interaction in NaTCDC micelles occur at 3.5 mM. These molecular changes observed in the CMC formation of NaTCDC were also characterized by an abrupt increase in viscosity between the concentrations of 2.5 and 3.5 mM (**Fig. 3**).

Changes in pH, ionic strength of the solution, solvent polarity, and temperature have been shown to have a marked effect on CMC (3). To determine the true CMC for NaGC, it must be completely in the salt form. Therefore, the pH of the system must be sufficiently high to ensure complete ionization. Whereas TCDC is completely ionized at pH > 2-4, NaGC is completely ionized only at pH > 8 (2). Therefore, the viscosity and infrared vibrational properties of NaGC were studied in 100 mM phosphate buffer at pH 8.0. Frequency changes in the CH_2 asymmetric stretch with increasing NaGC concentration followed a bell-shaped response (**Fig. 4**). Frequencies increased from 2935 to 2943 cm^{-1} upon increasing the concentration from 4 to 9 mM and declined to the baseline frequency at 14 mM of NaGC. Therefore, the critical micellar concentration for NaGC was determined to be 9 mM. Similar to TCDC spectra, at the CMC of NaGC a significantly greater intensity was observed for CH_2 symmetric and asymmetric stretches and the CH_3 asymmetric (2970 cm^{-1}) band emerged (**Fig. 5**). On the other hand, a more complex IR absorption pattern was seen for NaGC when it was prepared in 200 mM NaCl at pH 6.5. At this pH only about 70-75% of the NaGC is in the fully ionized state, producing at least two micellization stages. Increases in the frequency of the asymmetric CH_2 stretching bands indicated an initial CMC formation in the range of 6-10 mM and a second CMC range at the higher concentrations of 12-14 mM. Viscosity measurements of

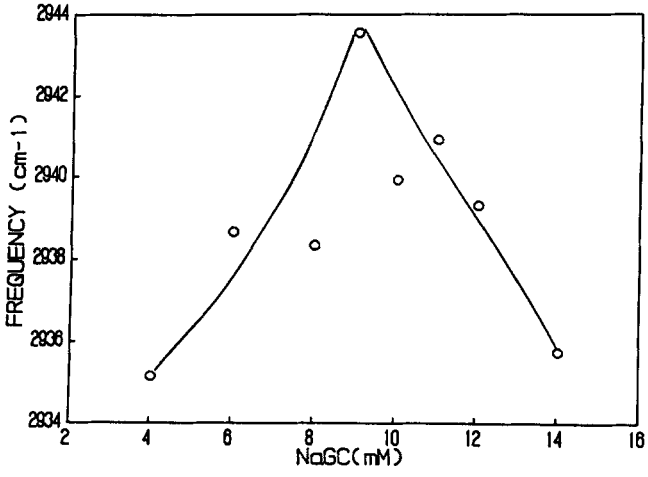

Fig. 4. Infrared absorbance maxima of the asymmetric CH_2 stretching bands of 4-14 mM NaGC in 100 mM Na_2HPO_4, at pH 8.0. CMC formation was noted by a maximal increase in frequency at 9 mM. The graph shows the average results from three experiments and the standard error was <4 cm^{-1}.

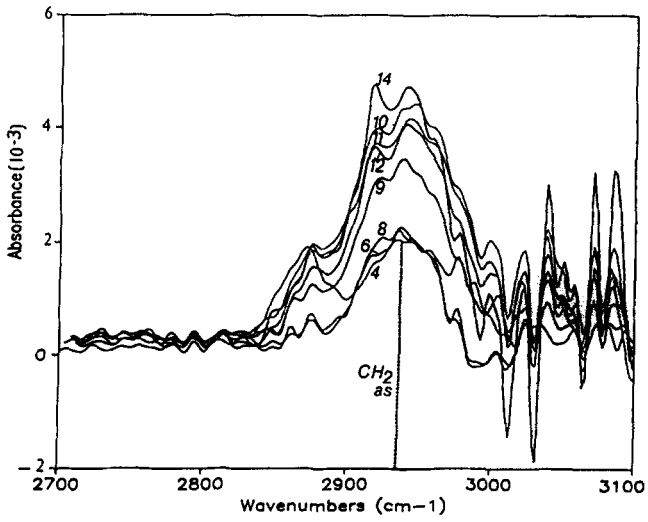

Fig. 5. IR spectra of 4–14 mM NaGC in 100 mM sodium phosphate, pH 8.0. Two major spectral changes occur in the region of CMC. 1) Frequency increases occur in asymmetric (~2935 cm^{-1}) stretches for concentrations 4–14 mM NaGC with a maximum at 9 mM. 2) The CH_3 asymmetric (2970 cm^{-1}) stretch NaGC with a maximum at 9 mM emerges and is most prominent at 9 mM NaGC, suggesting a tightly packed micellar structure.

NaGC indicated CMC formation by an abrupt increase in viscosity between concentrations 9–11 mM (**Fig. 6**).

DISCUSSION

We have studied the vibrational properties of the aliphatic bonds in bile salt molecules by FTIR spectroscopy and micellar growth properties by viscometry in order to understand the physical-chemical factors that lead to CMC formation and aggregation of bile lipids.

IR spectroscopy has allowed us to examine the mechanism of CMC formation. The initial stage of CMC formation of TCDC was achieved at 2.5 mM, where the CH_2 frequency shift reached a maximum (Fig. 2). Stable TCDC micelles continued to form up to 3.5 mM where a super critical micellar concentration (SMCS) was achieved and highly packed micelles were formed and the spectral intensity was at a maximum (Fig. 1). Spectral changes (both intensity and frequency) of NaGC were, however, more abrupt and occurred at 9 mM (Figs. 4 and 5). Values obtained by IR spectroscopy compared favorably with previously reported values (4–6). The CMC of NaTCDC has been determined to be 7 mM in water and 3 mM in 0.15 M Na^+ by using surface tension studies (4). An average CMC value of 2.4 mM is reported for NaTCDC which represents the mean of 48 different determinations (2). The CMC of NaGC has been determined to be 12 mM in water and 10 mM in 0.15 M Na^+ by using surface tension studies (4). The CMC values determined by surface tension measurements for both NaGC and NaTCDC in the presence of 0.15 M Na^+ correlate with our viscosity and IR measurements (this study) in both 200 mM NaCl and 100 mM Na phosphate buffer. Most methods for detecting CMCs of bile salts have required the presence of a "reporter" molecule. Although the addition of a reporter molecule may simplify the measurement of the CMC, the reporter molecule, by the nature of its shape, polarity, or charge, may perturb the nascent micelle and therefore change the true CMC of the bile salt. Previously we have shown that UV-VIS spectrophotometry can be used for the determination of the CMC of bile salts using bilirubin IXα monoglucuronide (BMG), a normal constituent of bile, as the reporter molecule (5). In the presence of BMG, abrupt changes in the UV-VIS absorption and second derivative spectra of BMG in TCDC were observed between 2.5 and 3 mM which were indicative of critical micellization.

The sharp increase in the slope of NaTCDC and NaGC in Figs. 3 and 6 implies that the viscosity of these bile salts increases rapidly with small changes in the bile salt concentration. This is consistent with the critical micellization process with cooperative interaction of monomers over a narrow concentration range (2), where a sudden rise in micellar size is indicated.

The interaction of the bile salt monomers in bile micelle formation is due to the aggregation of hydrophobic surfaces. In order to maximize van der Waals contacts between the alicyclic steroid on the convex hydrophobic side of the molecule, aggregation occurs in a back to back manner (7). Consequently, the observed frequency increases in CH_2 asymmetric (~2930 cm^{-1}) stretches can be used as an indicator for CMC formation. Continuous spectral changes in the alphatic bond stretching region of TCDC suggest that initial micellization occurs at 2.5

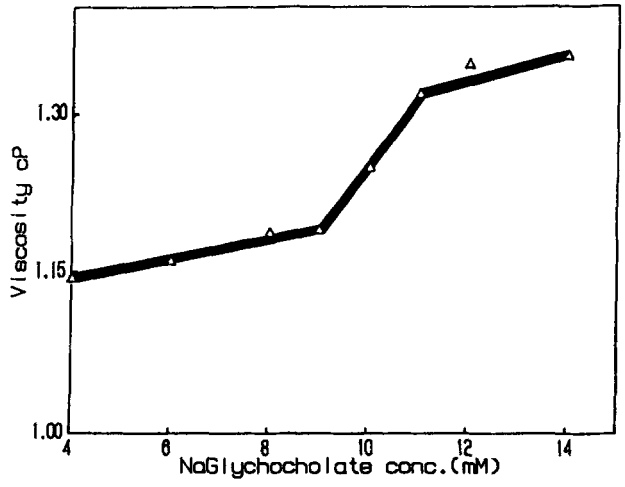

Fig. 6. Viscosity measurements of 4–14 mM NaGC in 200 mM NaCl. CMC formation is noted by an abrupt increase in viscosity between concentrations 9 and 11 mM.

mM. At SCMC of TCDC, however, the sudden increase in intensities of both aliphatic and sulfonate stretches, and especially the prominence of the CH_3 stretching region, reflected the maximal interaction of both the steroid skeletons and the polar head groups. The SCMC may represent the highest energy state of the bile salt micelles. This higher energy state then settles down with increasing bile salt concentration and micellar size, as strong hydrophobic interactions and conformational strains are gradually relaxed. Similar settling down phenomenon was also noted above the CMC of TCDC using UV-VIS spectroscopy (5).

We conclude that FTIR is useful in studying the mechanism of self-association of biliary lipids in aqueous solutions at the molecular level and correlates well with viscometric studies that are indicative of particle size. IR and viscometric studies of bile salt solutions and of model biles may be of therapeutic importance. Increases in viscosity of bile may contribute to the development of cholestasis in premature infants where the driving force for bile secretion may not be sufficient to oppose high intracanalicular viscosities. Thus, studies that can further characterize the intermolecular associations and viscous characteristics of bile may explain the factors that hinder the resolution of cholestasis.

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